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14. ABSTRACT

The purpose of this project is to generate the cell type-specific molecular blueprint of changes in gene expression following different types of noise exposure and their treatments, in the inner ear. To this end, we have (a) Established the hair cell (HC) and supporting (SC) cell-specific translatome of adult mouse inner ears; (b) Established the molecular changes induced by PTS-resulting noise exposure in HC, SC and whole inner ears, 6 and 24 hours after noise exposure and began the TTS analysis; (c) We identified critical differences in the response of male and female mice to noise, and obtained approval to modify Specific Aim 3 to focus on the response to Heat Shock profiling male and female mice, separately.

15. SUBJECT TERMS

Permanent threshold shift, Temporary threshold shift, Noise induced hearing loss, Ribotag, RNA-seq, hair cell, supporting cell, SAHA, Heat shock, sex differences

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1. INTRODUCTION

Noise induced hearing loss (NIHL) is a major health concern for the Department of Defense. Noise exposure often is inevitable, and may results in a permanent loss of hearing. Unfortunately, there are no treatments to prevent or reverse NIHL. As a first step towards designing targeted therapeutics, we suggested to generate mouse models which allow cell type-specific translatome analysis in the ear. These, in turn, will be used to analyze the genes expressed in the hair cells (HC) and supporting cells (SC) of adult mice before and after different types of noise exposure as well as pre-conditioning treatments, which in mice, can ameliorate NIHL. Here we report our progress over the third year of the project, in which (a) HC and SC from adult mice were sequenced and their expression patterns were compared and analyzed; (b) we established the molecular cell type-specific blueprint following PTS-inducing noise exposure; (c) we identified differences in the molecular response to PTS- and TTS-inducing noise exposure; and (d) we identified critical differences in the response of male and female mice to noise exposure, which lead to modification of Specific Aim 3 (manuscript in preparation, data shared at the ARO in an oral presentation).

2. KEYWORDS

Permanent threshold shift, Temporary threshold shift, Noise induced hearing loss, Ribotag, RNA-seq, Hair cell, Supporting cell, SAHA, Heat shock, Sex differences.

3. ACCOMPLISHMENTS

<u>Specific Aim 1</u>: To determine the OHC- and SC-specific transcriptional and signaling cascades activated in vivo in response to PTS-inducing noise exposure

- <u>Major Task 1</u>: To establish the OHC- and SC-specific translatome of adult mouse inner ears. Progress by subtasks:
    i. Obtain ACURO approval following UMSOM IACUC approval – complete.
    ii. Mouse crosses and tissue harvesting – complete.
    iii. Tissue processing – complete, polysome IP – complete, submission of samples for RiboTag-seq – complete; RiboTag-seq – complete**.**

- <u>We identified 410 genes with a significantly higher expression in OHC</u> compared with SC and the rest of the inner ear. Many of the genes have not been previously described and/or studied in relation to inner ear development/function. Within the top 10 genes we find: Klhdc7b, **Strc**, Dlec1, Btn2a2, **Slc26a5**, **Gfi1**, **Lmod3**, **Ocm** and **Lhfpl5**. Of these

ten genes, six (marked in bold) are known to be expressed in hair cells and when mutated to cause a deafness phenotype. Many additional deafness genes are included in the list, indicating that their neighboring genes are highly likely to have a critical role in maintenance of the hair cells. Importantly, Scl26a5, which was used to pull down the ribosomes in this assay is in the top 5 most enriched genes, validating our approach. A *cis*-regulatory analysis identified IKZF2 and GFI1 as the likely most prominent regulators of the adult OHC-translatome. While IKZF2 role in hearing has yet to be published, in a separate project we identified IKZF2 as a critical regulator of OHC electromotility, further validating our findings. Two manuscripts are now in preparation.

- We also identified 282 SC enriched genes. Much less is known about the adult supporting cell translatome. The following genes were within the top 10 most enriched SC genes: Igfbp6, Bpifa1, Timp4, Aldoc, Ascbg1, Gldc, Egfl6, Plbd1, Mlc1 and Slc25a18. From these, Bpifa1 has been previously associated with susceptibility to otitis media (PMID 25765466), Timp4 with worse outcomes after myocardial infarction in mice (PMID 20516072), Aldoc was previously described as expressed in the ear but at a very low resolution and only in neonatal mice (PMID 24475166), and Slc25a18 has a role with Glutamate transport. Importantly, Sox2, which was used to pull down the ribosomes, is within the top 30 enriched genes. Its lower location compared to Slc26a5 highlights how little we know about SC in the adult mouse. Also, many of the enriched genes, do follow even in the early postnatal mouse a pattern of increasing expression in the non-HC population based on the SHIELD further increasing our confidence level with regards to their specific expression in adult tissues. Finally, a *cis*-regulatory analysis of the SC-enriched genes pointed to SOX2 and REST as their likely key regulators.

- Specificity of cre-drivers. An important step in the data analysis of each experiment is careful characterization of its individual components. Here we validated the specificity of the cre-drivers for OHC and SC (see **Figure 1** and **Table 1**). The OHC-cre, driven by the prestin locus was found to be specific for OHC (the red reported is shown only in OHC, **Figure 1**) and HC and OHC transcripts are enriched in the immunoprecipitate while all other transcripts are depleted (**Table 1**). The SC-cre, driven by the Sox2 gene was found to induce recombination both in SC and in glia (see **Figure 1** and **Table 1**). This result was not anticipated, however, it does not take away from the validity of the data. We now know that SC transcripts in this dataset include also glia.

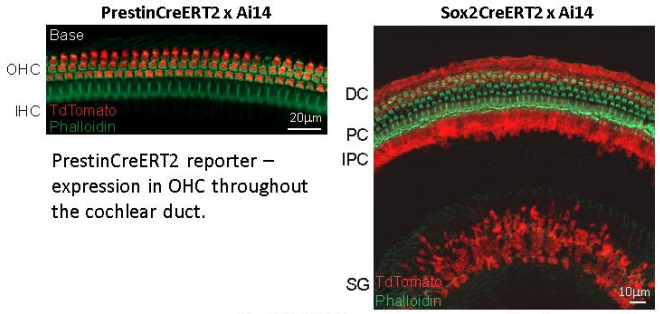


Figure 1 Validation of OHC and SC Cre drivers*. PrestinCreERT2 (cre-driver for OHC, left panel) and Sox2CreERT2 (driver for SC, right panel) were crossed with a mouse that expresses a red fluorescent protein in the presence of cre-recombinase (Ai14).*

Table 1 - Enrichment analysis of immunoprecipitated transcripts from the Prestin and Sox2 cre mice.

Cell Types	Gene Symbol	$Rpl22^{HA/+}$;$Prestin^{CreERT2/+}$			$Rpl22^{HA/+}$;$Sox2^{CreERT2/+}$		
		Input	IP	Enrichment /Depletion (Log2)	Input	IP	Enrichment /Depletion (Log2)
Outer Hair Cells	Slc26a5	134	559	2.06	414	144	-1.53
	Ocm	793	5624	2.83	2061	1316	-0.65
Hair Cells	Gfi1	53	200	1.91	136	44	-1.63
	Strc	128	763	2.57	388	141	-1.47
Supporting Cells	Sox2	336	186	-0.85	544	2984	2.46
	Jag1	444	431	-0.04	847	2729	1.69
Neurons	Tubb3	1453	608	-1.26	3407	3820	0.16
	Dlg4	1165	979	-0.25	3984	5779	0.54
Glia (Astrocytes)	Gfap	165	101	-0.71	1212	6639	2.45
	Aldh1l1	327	209	-0.64	1198	2422	1.02
Mesenchyme	Pou3f4	2023	993	-1.03	5501	1523	-1.85
	Etv1	546	242	-1.18	1464	728	-1.01

- Major Task 2: To determine the OHC- and SC-specific transcriptional and signaling cascades activated in response to PTS-inducing noise injury. Progress by subtasks:
    i. Mouse crosses, noise exposure, tissue harvesting, histological analysis, ABR and DPOAE measurements. Complete.
    ii. Tissue processing – complete.
    iii. Data analysis – largely reported last year.
    iv. Validation experiments – polysome IP to be used for RT-qPCR – complete. Tissue harvesting for immunohistochemistry and in situ hybridization - complete. Validation experiments to be performed with NanoString in the last quarter of year IV of the project.

Future plans:

- Validation of select genes by in-situ hybridization and/or immunohistochemistry
- Possible interrogation of select mouse mutants for these genes (as part of this or future applications)

Specific Aim 2: To determine the OHC- and SC-specific signaling cascades activated in vivo in response to otoprotective interventions.

This Aim was designed to define the cell type-specific molecular blueprint of interventions that may ameliorate NIHL. We planned to characterize TTS-inducing noise exposure, heat shock and restraint stress. In our previous annual reports we discussed the rationale for maintaining the TTS and Heat Shock. The heat shock goal was transferred to a modified Specific Aim 3 that is also sex-specific.

TTS-inducing noise exposure: crosses, calibration, validation cytocochleograms, noise exposure, tissue harvesting, polysome IP and RNA extraction was completed both for the OHC and SC crosses. Sequencing (of tissue obtained from males and females, combined) is complete. Analysis – ongoing.

Preliminary Results

In the analysis of tissue obtained from whole ears (IP tissue has been sequenced but analysis is ongoing) we identified 12 clusters of genes that are distinct in their behaviors (**Figure 2**). For

example, **cluster 1** consists of 176 genes that are uniquely downregulated following TTS-inducing noise exposure. In contrast **cluster 3** consists of 49 genes that are uniquely upregulated after TTS-inducing noise exposure. As TTS-inducing noise exposure has also been shown to be otoprotective from PTS, chemotherapy and aminoglycoside exposure – these genes are well positioned to be candidate otoportective genes. Interestingly, within this group we find DFNA5, which in its absence mice and human suffer from sensorineural hearing loss, further supporting our hypothesis. **Cluster 2** consists of genes that increase within 24 hours of exposure to PTS inducing noise exposure but not after TTS inducing noise exposure. This cluster is statistically significantly enriched for genes that encode proteins of the innate immune response (p-value 3.65e-06 and corrected p-value 0.009) (see **Figure 3**). The significance of this finding is that one of the defining features differentiating between the response to PTS and TTS is activation of the innate immune response, a finding with a clear translational significance.

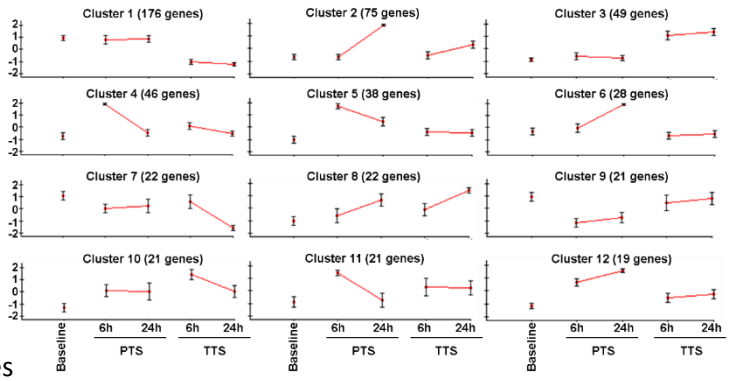


***Figure 2 cluster analysis of the differences between the molecular response to PTS and TTS**. In each graph the first point represents the baseline values, followed by expression at 6 and 24 hours, first in PTS and then in TTS. Y axis - log2 transformed.*

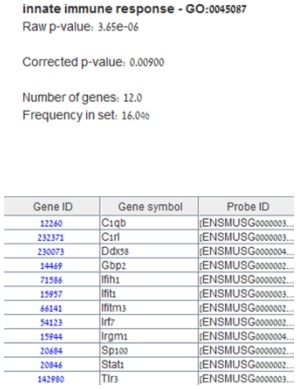


Figure 3 Innate immune response genes are enriched in cluster 2.

An interesting observation as part of the results of both specific aims 1 and 2 is an activation of 'immediate early genes' also known as 'stress genes' in the samples exposed to noise (e.g., Fos, Jun, Hspa1a). The increase in gene expression seen at 6 hours decreases dramatically for some of the genes at 24 hours and for other genes persists (see **Figure 4**). A recent publication (Nature Methods, Vol 14 No.10, October 2017) showed that this particular group of genes is activated in a non-specific manner following tissue dissociation (**Figure 4**). This finding further strengthens the importance of our chosen approach for interrogating gene expression following noise exposure – as cell sorting or even just tissue dissociation for traditional single-cell RNA-seq is likely to invoke numerous changes in gene expression that would specifically mask changes of interest in this type of experiments.

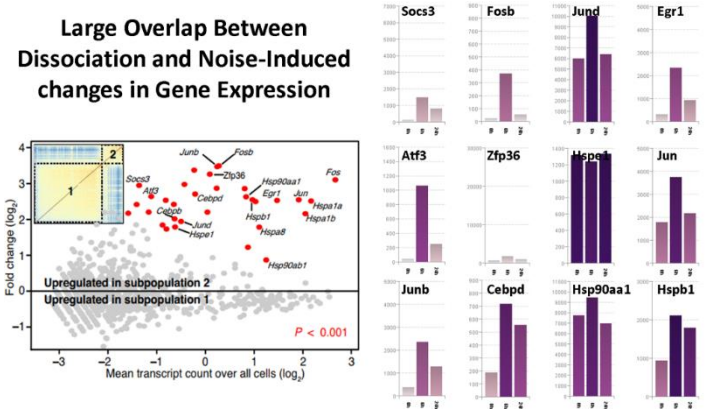


Figure 4 Overlap between dissociation and noise-induced changes in gene expression

<u>Specific Aim 3</u>: Following the in-person review in the DoD this summer, specific aim 3 was redesigned to allow us to both study

the response to Heat Shock with and without noise exposure as originally planned as well as obtain all tissue separately from male and female mice. This was presented, submitted to and approved by the DoD. We are currently in the process of tissue collection and hope to complete that stage by March 2018.

Key research accomplishments

- Identification of OHC and SC unique genes and their regulators.
- Molecular description of the response to PTS in OHC, SC and whole inner ear.
- Molecular description of the response to TTS.
- Key differences between the response to TTS and PTS.
- Critical differences in the response to noise between males and females.

Conclusion

The results of the cell type-specific molecular changes as a result of PTS-inducing noise exposure will set a foundation for rationale design of drugs to treat NIHL. This is critically important for the military, hearing loss and tinnitus are a principal cause for disability in the DoD and to date there are no efficient preventative or curative treatments. Maximal benefit from our molecular data is pending the TTS-inducing noise exposure dataset, as the latter will allow to distinguish protective changes in gene expression from those that represent a detrimental effect on hearing. Indeed, our initial analysis shows that at least one deafness causing gene (DFNA5) is upregulated specifically after TTS-resulting noise exposure. In addition, our results demonstrate the feasibility of using cell type-specific molecular changes as a readout in future studies to protect from NIHL, and the advantage of the RiboTag approach over the single-cell or dissociation-based approaches. With our current results we anticipate being able to develop a molecular panel predictive of type of noise injury that could be used as a fast-readout to screen for the effect of therapeutics. Our identified differences in the physiologic response to noise between male and female mice are important, not only for the experimental design of future studies, but as both men and women now serves in the army in positions where they suffer from NIHL, and we should therefore design treatments that will be efficacious in both sexes. In addition, the new structure of specific aim 3 will enable us to understand, at a molecular level, the differences between the response of male and female mice to noise.

Publications, abstracts, and presentations

- Three manuscripts are in the final stages of preparation for publication: the kit comparison manuscript, differences in the response to noise between male and female mice, and the identification of IKZF2 as a key regulator of the OHC translatome.
- The findings about the sex-differences, which are critically important for experimental design were shared at the ARO 2017 Mid-Winter Meeting (pre-publication, oral presentation)
  Mitra S, Drake V, Margulies Z, Milon B, Song Y, Depireux D and Hertzano R (2017) The impact of sex on the response to noise and otoprotective therapies against acoustic injury in mice; Association for Research in Otolaryngology, Baltimore, MD, USA.
- The findings of the kit-comparison work were shared at the ARO (pre-publication, oral presentation)
  Song Y, Milon B, Ott S, Zhao X, Sadzewicz L, Mahurkar A and Hertzano R (2017) RiboTag-Seq: a comparative analysis of library prep approaches for sequencing low

input translatome samples; Association for Research in Otolaryngology, Baltimore, MD, USA.

Opportunities for training and professional development

Training or fellowship awards:

Sunayana Mitra, PhD, participated in the Association for Research in Otolaryngology meeting, as well as in the EARssentials course at the NIH.

Beatrice Milon, PhD, participated in the Association for Research in Otolaryngology meeting.

Ryan Casserly, MD, worked in the Hertzano laboratory in a 5-month full time research rotation to characterize the differential response to noise between male and female mice.

Laboratory meetings - since obtaining funding from the DoD the entire Hertzano laboratory engages in in-depth study of current literature and techniques to study NIHL and has been increasing their knowledge and experience through laboratory meetings and journal clubs. The team has also trained two additional laboratories in the department (laboratories of Drs. Ahmed and Riazuddin) who now focus some of their work on NIHL. In addition, this past year, we developed a new series of laboratory meetings named H&H (Hearing and Hormones) attended by Dr. Jessica A. Mong (a neuroendocrinologist with a focus on sex differences in the brain) and our group.

Professional development

All members working on the DoD project participate in laboratory meetings, the translational Auditory and Vestibular research day, the Association for Research in Otolaryngology mid-winter meeting.

Inventions, patents and licences

Nothing to report

Reportable outcomes

Nothing to report

Other achievements

Nothing to report.

References

4. IMPACT

- Our work and its presentation in scientific meetings has significantly increased awareness in the ear field to cell type specific molecular analysis using the RiboTag mice, and better approaches for sequencing of their translatomes.
- Our work and its presentation in scientific meetings has brought sex differences in the response to noise exposure to the awareness of the ear field scientific community and we believe will have broad impact on study designs.
- The results of our work will form the foundation for new rationale design of therapeutics to prevent noise induced hearing loss.

5. **CHANGES/PROBLEMS**

We had a delay in the breeding efficiency this year and therefore the baseline heat shock experiments are done on C57BL/6 and not the mixed background. However, with improvements to the sequencing protocol, we can now catch up over the year thanks to requiring less mice per biological replicate.

All changes in structure and expenditures have been reported in the in-person review (within the presentation) and are within the limits of the budget of the project.

No significant changes were made to biohazards.

6. **PRODUCTS**

Nothing to report.

7. **PARTICIPANTS AND OTHER COLLABORATING ORGANIZATIONS**

Individuals who work on the project

Name	**Ronna Hertzano**
Project Role	**PI**
Researcher identifier	
Nearest person month worked:	**2**
Contribution to the project	**Overall responsibility for the proposal and all aspects of the research program including: hiring and training personnel, ensuring quality of data, interpretation of data, oversight of methods, administrative responsibility and reporting to the DoD.**
Funding support	**NIH R01, DC013817; Action on Hearing Loss. G65_Bowl; NIH R01, DC003544; Hearing Health Foundation – HRP support for gEAR.**

Name	**Didier Depireux**
Project Role	**Co-I**
Researcher identifier	
Nearest person month worked:	**1.2**
Contribution to the project	**Oversight of the noise exposure protocols, ABR and DPOAE setup and measurements; Discussion and analysis of Male/Female data.**
Funding support	**MII, Translational Research in Hearing Foundation, Capita foundation, NIH/NIDCR**

Name	**Ran Elkon**
Project Role	**Co-I**
Researcher identifier	
Nearest person month worked:	**1.2**

Contribution to the project	**Data analysis and study design**
Funding support	

Name	**Yang Song**
Project Role	**Analyst**
Researcher identifier	
Nearest person month worked:	**1.2**
Contribution to the project	**Data analysis**
Funding support	

Name	**Beatrice Milon**
Project Role	**Research Supervisor**
Researcher identifier	
Nearest person month worked:	**6 months**
Contribution to the project	**Study design, tissue collection, schedule oversight, training Yoko Ogawa, RiboTag IP, RNA analysis, cytocochleograms**
Funding support	**NIH R01, DC013817**

Name	**Yoko Ogawa**
Project Role	**Post Doctoral Fellow**
Researcher identifier	
Nearest person month worked:	**5 months**
Contribution to the project	**Tissue collection, cytocochleograms, validation (currently setting up), animal care**
Funding support	

Name	**Ryan Casserly**
Project Role	**Resident**
Researcher identifier	
Nearest person month worked:	**3 months**
Contribution to the project	**Characterized the sex differences in hearing**
Funding support	

Name	**Sunayana Mitra**
Project Role	**Post Doctoral Fellow**
Researcher identifier	

Nearest person month worked:	**12 months**
Contribution to the project	**Performs all noise exposures, hearing measurements, pre-conditioning, tissue collection, animal care, assists with writing animal protocols**
Funding support	

Changes in the other support of the PI/ other key personnel:

- No significant changes.

8. SPECIAL REPORTING

QUAD CHART

Towards a molecular understanding of noise induced hearing loss

Log number: MR130240

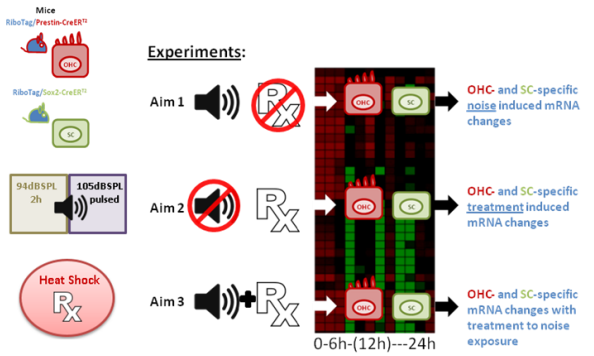
PI: Ronna Hertzano **Org:** University of Maryland School of Medicine **Award Amount:** $1,500,000

Study/Product Aim(s)

• Characterization of the translatomes of adult outer hair cells (OHC) and supporting cells (SC) at baseline.
• Identification, characterization and validation of the cell type-specific translational changes secondary to noise trauma that induces a permanent in comparison to a temporary hearing loss.
• Identification of the cell type-specific signaling cascades activated during successful treatment of noise induced hearing loss in mice (e.g. Heat Shock).
• Define at least two key signaling cascades that could be targeted to develop new therapeutic interventions to prevent and treat noise induced hearing loss.
•Determine the sex-specific differences in the molecular response to noise exposure.

Approach

We established animal models that allow cell type-specific RNA extraction from adult mouse inner ears, to determine the cell type-specific translational changes at different time points following noise exposure with and without treatment, compared with the baseline translatomes prior to exposure. Identified key regulatory pathways will be validated using real time RT-PCR.



Timeline and Cost

Activities CY	14-15	15-16	16-17	17-18
To establish the OHC- and SC-specific translatome of adult mouse inner ears				
To determine the OHC- and SC-specific transcriptional and signaling cascades activated in response to PTS-inducing noise injury				
To determine the OHC- and SC-specific signaling cascades activated in vivo in response to otoprotective interventions				
To evaluate the OHC- and SC-specific signaling cascades affected *in vivo* in mice, in males and females separately, to PTS and TTS noise, as well as Heat Shock, and PTS noise and Heat Shock combined				
Estimated Budget ($K)	$400	$400	$400	$300

Updated: (October 26, 2017)

Goals/Milestones

CY14-16 Goal – OHC- and SC- translatomes at baseline
☑ Identification and characterization of the OHC and SC-specific translatomes

CY15-16 Goal – Defining the inner ear translatome in response to noise trauma
☑ Identification of the key signaling cascades initiated by noise trauma
☑ Identification of differences in the response of male and female mice to noise

CY16-17 Goal – In vivo dissection of cell type-specific changes in response to treatment and pre-treated noise exposure
☑ Identification and characterization of the signaling pathways induced by otoprotective interventions from noise induced hearing loss
☐ Comparison of the cell type-specific molecular changes in response to PTS- and TTS-inducing noise exposure
☑ Improved approaches for Ribotag seq using fewer mice per experiment

CY17-18 Goal – Defining the inner ear translatome in response to noise trauma
☐ Identification of at least two new pathways for generating new interventions to prevent and treat noise induced hearing loss
☐ Comparison of the cell type-specific molecular responses to PTS-inducing noise with and without protective interventions.
☐ Identify some of the sex-specific molecular changes in response to noise.

Budget Expenditure to Date
Projected Expenditure: 1,500,000
Actual Expenditure: 1,144,731(as of September 2017)

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